Enhancement of Heparin-Binding Ability of Fibronectin by S-Carboxamide-methylation

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Human plasma fibronectin (FN) was reduced and carboxamide-methylated, and its binding ability to several matrices was analyzed in vitro. The binding of S-carboxamide-methyl (Cam)-FN to heparin-Sepharose was not influenced by either 4 M urea, 0.5 M NaCl or 0.5% heparin, but was disrupted by the coexistence of urea and NaCl or heparin. S-Cam-FN, compared with intact FN, obviously had a more potent ability to bind heparin, while it had little or no binding ability to gelatin, fibrin and thrombin-stimulated platelets. A conformational change of S-Cam-FN by heparin-binding has been proposed as a possible mechanism from the result of circular dichroic spectrum measurement.

Keywords — fibronectin; alkylation; carboxamide-methylation; heparin; heparin-binding; affinity chromatography; circular dichroic spectrum

Introduction

Recently, great interest has developed in the role of chemical modification in developing new or improved functionality of protein. We have reported that S-carboxamide-methylated (Cam) human immunoglobulin (Ig) G, like 2-(E-2-alkenylamino)ethyl carbamoylmethyl sulfides, showed new pharmacological activities, though the native IgG did not. It was suggested that the carboxamide-methylation of S-S bonds was essential to developing these functionalities.

Plasma fibronectin (FN) is a disulfide-bonded dimer with intramolecular disulfide bonds. It has binding activities for cell surface, gelatin, heparin, fibrin and other molecules, which are located in different domains of the molecule. In this paper, we describe our primary results in attempting to develop an improved functionality of human plasma FN by S-carboxamide-methylation.

Materials and Methods

Materials — Human plasma FN preparation was kindly supplied from Green Cross Corporation, Ltd. (Osaka, Japan) and further purified by the chromatography on gelatin-Sepharose 4B. Sepharose and Sephacryl media were obtained from Pharmacia LKB Biotechnology, Uppsala, Sweden; Bio-Gel A-150 m and

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<th>lane</th>
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<tr>
<td>kDa</td>
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<td></td>
<td>43</td>
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Fig. 1. SDS-PAGE of S-Cam-FN
Sample was added to a solution containing 2% SDS, and the resultant mixture was analyzed by SDS-PAGE, using a 5% polyacrylamide gel without reducing agent. Protein was detected with Coomassie brilliant blue. Lane 1, S-Cam-FN and 2, molecular weight standards.
molecular weight standard (SDS-PAGE standard, high) were obtained from Nippon BIO-RAD Laboratory Ltd., Tokyo, Japan; Na\textsuperscript{125}I from Amersham International plc, Buckinghamshire, England, and heparin, fibrinogen and bovine serum albumin (BSA) from Sigma Chemical Co., St. Louis, Mo. U.S.A. All other reagents and solvents used were of analytical grade.

**Preparation of S-Carboxamidemethyl (Cam)-FN** — Purified FN was completely reduced and carboxamidemethylated as described for immunoglobulin.\(^6\) Briefly, the FN (0.023 mM) was denatured in 6 M guanidine hydrochloride, 0.5 M tris-HCl (pH 8.1) and 2 mM ethylenediamine tetraacetic acid (EDTA) at 50 °C for 30 min under bubbling with N\textsubscript{2} gas, reduced with 70 mM dithiothreitol at 50 °C for 4 h under N\textsubscript{2} bubbling and then alkylated with 140 mM iodoacetamide at 50 °C for 20 min in the dark. S-Cam-FN was isolated from the reagents by gel filtration on Sephacryl S-300 using 50 mM tris-HCl (pH 7.4) as a solvent. S-Cam-FN gave a dimer of similar but not identical polypeptide bands of molecular weight of about 250000 in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)\(^7\) without reducing treatment (Fig. 1). Amino acid analysis of the hydrolysates with 6 N HCl at 110 °C for 24 h gave 1.2 mol% of cystine in FN, and 2.3 mol% of carboxymethylcysteine and no cystine in S-Cam-FN. The amino acid composition of S-Cam-FN was almost the same as that of intact FN, except for the carboxymethylcysteine and cystine. Furthermore, the disappearance of disulfide bonds was proved by circular dichroic (CD) spectra (Fig. 4). In this paper, S-Cam-FN refers to the mixture of S-Cam-FN-A chain and -B chain. According to the method of Heinrikson,\(^8\) the FN (0.023 mM) was denatured in 6 M guanidine hydrochloride, 0.25 M tris-HCl (pH 8.6), 3.3 mM EDTA and 25% (v/v) acetonitrile for 1 min under bubbling with N\textsubscript{2} gas, reduced with 70 mM 2-mercaptoethanol at 50 °C for 1 h under N\textsubscript{2} bubbling and alkylated with 140 mM methyl p-nitrobenzenesulfonic acid Na (in 0.5 ml acetonitrile) at 37 °C for 2 h. S-Methylated (Me)-FN was isolated from the reaction mixture by dialysis against 4 M urea in 50 mM tris-HCl (pH 7.4). S-Met-FN was insoluble in aqueous solution without urea. The FN was radioiodinated with a carrier free Na\textsuperscript{125}I by a modified chloramine-T procedure as described.\(^9\) The specific activity of 125I-FN was 1 μCi/μg.

**Binding Assay** — S-Cam-FN was analyzed as to its binding to heparin, gelatin and fibrin by means of affinity chromatographies using heparin-Sepharose CL-6B, gelatin-Sepharose 4B and fibrin-Sepharose 4B, respectively.\(^5\) Samples (about 0.5 mg) were applied to 1.5 ml columns of Sepharose. Sample applications and elutions were carried out at 0.25 ml/min at 20—25 °C.

The binding activity of S-Cam-FN to thrombin-stimulated platelets was measured as follows.\(^9\) Platelets were isolated from citrated rat blood by differential centrifugation and gel filtration on Bio-gel A-150 m. Platelets were suspended in 50 μl of a modified Tyrode's buffer (pH 7.4) containing 2 mM MgCl\textsubscript{2}. Then 5 μl of thrombin solution was added to the suspension immediately after 200 μl of 10 mM sodium phosphate (pH 7.4)-0.15 M NaCl (PBS) or unlabeled ligand and 50 μl of 125I-FN in PBS containing 1% BSA. Incubation was performed at 37 °C, and 30 min later 50 μl aliquots were layered onto 300 μl of 20% sucrose and centrifuged at 12000 rpm for 5 min. The resultant precipitate was counted, and the fibronectin bound was calculated from the specific activity of the ligand.

**CD Spectrum Measurement** — According to the method of Österlund et al.,\(^10\) the spectra were measured on a Nippon Bunko Model J-500A spectropolarimeter under the following conditions; temperature: 25 °C, cell path length: 2 mm, scan rate: 50 nm/min and scan time : 8, sample concentration : 0.1 mg/ml for FN solutions, solvent : 50 mM sodium phosphate buffer (pH 7.4). Appropriate blanks were subtracted for each sample, and molecular ellipticities \((\text{deg} \cdot \text{cm}^2 \cdot \text{dmol}^{-1})\), \([\theta]_\lambda\) were calculated with an average molecular weight of 450 000 according to the standard formula.\(^11\)

**Estimation of FN Concentrations** — Concentrations of FN solutions used were estimated spectrophotometrically, assuming a value of A (1%, 1 cm) = 12.8 at 280 nm.\(^12\)
Results

Binding to Gelatin, Fibrin and Platelet

FN bound to gelatin-Sepharose 4B in 50 mM tris-HCl (pH 7.4) and was eluted with 4 M urea, while S-Cam-FN did not bind to gelatin-Sepharose (Fig. 2 A, B). FN bound to fibrin-Sepharose 4B in physiological salt solution and was eluted with 8 M urea, while about one-third of applied S-Cam-FN bound to fibrin-Sepharose 4B under the same physiological conditions (Fig. 2 C, D).

Table I. Effect of S-Cam-FN on Binding of ¹²⁵I-FN to Thrombin-Stimulated Platelets

<table>
<thead>
<tr>
<th>Added samples</th>
<th>Final concentration</th>
<th>¹²⁵I-FN binding per 10⁷ platelets (pg, means ± S.E., n = 3)</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>—</td>
<td>721 ± 199</td>
<td>—</td>
</tr>
<tr>
<td>FN</td>
<td>0.3 mg/ml</td>
<td>60 ± 28</td>
<td>92</td>
</tr>
<tr>
<td>S-Cam-FN</td>
<td>0.3 mg/ml</td>
<td>728 ± 98</td>
<td>0</td>
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¹²⁵I-FN at a final concentration of 1.0 nM and sample were added to 0.5 × 10⁶ thrombin-stimulated (2 units/ml) platelets. Binding was measured after a 30-min incubation at 37 °C.
Fig. 3. Affinity Chromatography of FN and S-Cam-FN on Heparin-Sepharose CL-6B
Column: (A-E) heparin-Sepharose CL-6B, (F) Sepharose CL-6B. Solvent: (A, B, E, F) Tris (50 mM tris-HCl, pH 7.4) → NaCl (0.5 mM NaCl) → NaCl + urea (NaCl in 4 M urea), (C) Tris → Heparin (0.5% heparin in Tris) → NaCl + urea, (D) (E) in urea.

a) Absorbance was corrected for heparin.
The effect of FN and S-Cam-FN on binding activity of $^{125}$I-FN to thrombin-stimulated platelets was investigated. At the concentration of 0.3 mg/ml, FN competed completely with $^{125}$I-FN for binding to platelets, while S-Cam-FN did not. It was indicated that S-Cam-FN did not bind to stimulated platelets (Table I).

**Binding to Heparin**

Both FN and S-Cam-FN bound to heparin-Sepharose CL-6B when the samples were applied and chromatographed in 50 mM tris-HCl (pH 7.4). The bound FN was eluted with 0.5 M NaCl, but the bound S-Cam-FN was not. The bound S-Cam-FN could be eluted with 0.5 M NaCl in 4 M urea (Fig. 3 A, B), but not with 4—8 M urea, 0.5—2 M NaCl, 1 M propionic acid (pH 1.5), 0.1 M NaHCO$_3$ (pH 9.1) and 3 M sodium trichloroacetic acid (pH 7.4) (data not shown). Also, the bound S-Cam-FN could be eluted with 0.5% heparin in 4 M urea, but not with only heparin (Fig. 3 C, D). FN, dialyzed against the starting buffer after treatment with 6 M guanidine-HCl, showed almost the same behavior as intact FN in the heparin-Sepharose CL-6B column. S-Cam-FN was not retained on a column of Sepharose CL-6B (Fig. 3 E, F). FN, S-Cam-FN and S-Me-FN bound to heparin-Sepharose CL-6B even when the samples were applied in 4 M urea. The bound FN was eluted with 0.5 M NaCl (Fig. 4 A), but the bound S-Cam-FN and S-Me-FN were not. They could be dissociated with 0.5 M NaCl in 4 M urea from the columns (Fig. 4 B, C). Therefore, S-alkyl-FN had a more potent ability to bind heparin than FN.

**CD Spectrum**

S-Carboxamidemethylation of FN caused a disappearance of a positive band at 227 nm (due to disulfide)$^{10,13}$ and an increase of negative ellipticity at 215 nm (due to $\beta$-structure)$^{10,13}$ in CD spectra between 200 and 250 nm (Fig. 5). The CD spectrum was changed by an addition of heparin: CD ellipticities between 200 and 250 nm varied to more negative values. These results indicate that heparin induces a conformational change of S-Cam-FN. An addition of urea to the mixture of S-Cam-FN and heparin decreased the negative CD ellipticities between 220 and 250 nm (Fig. 5).

**Discussion**

The reduction and carboxamidemethylation of FN resulted in the enhancement of binding ability to heparin. The decrease or disappearance of binding ability to gelatin, fibrin, and stimulated platelets was also derived from this modification of FN.
There are two distinct heparin-binding sites with differing affinity on each FN chain.\textsuperscript{5a,b} FN contains 29 intrachain disulfide bonds, in addition to two interchain disulfides and two sulf-hydryls per chain. Of these, 10 are located in 5 type I homology sequences of the amino-terminal heparin (lower affinity)- and fibrin-binding domain; 12 in 4 type I and 2 type II homology sequences of the gelatin-binding domain; 7 in 3 type I homology sequences of carboxyl-terminal fibrin-binding domain. The major heparin (higher affinity)-binding domain is in a region consisting of type III homology sequences which contain no disulfide group.\textsuperscript{14} Both bindings of intact FN and of isolated heparin-binding fragments to heparin are disrupted by 0.25—0.5 M NaCl, which implied that the interaction is only electrostatic via sulfate anion. In fact, the heparin-binding domains show higher isoelectric points of 8.2—8.9 than the other domains in the FN molecule.\textsuperscript{5b} The binding of S-Cam-FN to heparin-Sepharose was not influenced by either denaturant, high salt solution or heparin, but was inhibited by the coexistence of urea and NaCl or heparin. It seems likely that the binding is not only electrostatic.

This finding may be interpreted in light of the following evidence and supposition. Physical studies of FN suggest that it contains several globular domains connected by flexible regions;\textsuperscript{15} presumably, these globular regions are stabilized by disulfide bonds. FN probably exists in solution in a folded, but at least moderately elongated, conformation.\textsuperscript{15,16} The fragments containing disulfide bond-rich type I and type II homology sequences, which were reduced under denaturing conditions, transformed irreversibly into a more unfolded and extended structure than that in the native state.\textsuperscript{13,16 b} Therefore, the reductive cleavage and subsequent carboxamidemethylation of FN may cause the first conformational change in the protein leading to increased exposure of a positively charged sequence within a localized area of the molecule so that the basic residues are more accessible for interaction with heparin. It is noteworthy that disulfide-rich type I and type II homology sequences contain much more basic amino acids than type III homology sequences.\textsuperscript{14}

S-Me-FN, like S-Cam-FN, strongly bound to heparin-Sepharose. However, the binding of the reduced and carboxymethylated FN, as well as intact FN, to heparin-agarose was disrupted by 0.5 M NaCl.\textsuperscript{17} The carboxamide group is electrostatically neutral at neutral pH, but the carboxyl group charges negatively. It seems that at least part of the positively charged sequences in carboxymethyl FN were neutralized by newly
generated carboxyl groups. Therefore, it seems likely that the heparin binding sites on S-Cam-FN and S-Me-FN molecules are different from those of the intact FN.

It is interesting that a cell adhesive glycoprotein, vitronectin, also contains a buried and inducible heparin-binding site within the molecule.\(^{18}\)

It is also possible that heparin might induce the second change in the conformation of S-Cam-FN, because the presence of urea was necessary to elute S-Cam-FN retained on heparin-Sepharose. This conformational change of S-Cam-FN by heparin was confirmed by CD spectra measurements: CD ellipticities of S-Cam-FN between 200 and 250 nm varied to more negative values by an addition of heparin. Although the mechanism of a conformational change remains elusive, one interpretation is that S-Cam-FN may self-associate or fold up to bury its heparin binding site so that the surrounding of the site becomes highly hydrophobic.

It was reported that heparan sulfate\(^{10}\) and heparin\(^{19}\) induced changes in the conformation of FN and that FN polymerization was enhanced by heparin.\(^{20}\) S-Cam-FN might have more self-association sites than intact FN as a result of alternating overall positive and negative changes in adjacent domains for electrostatic linking of chains.

FN binds to cell by the interaction of Arg-Gly-Asp (RGD) sequence with a cell surface glycoprotein complex,\(^{21}\) and to gelatin and fibrin via \(\varepsilon\)-(\(\gamma\)-glutamyl)-lysine bonds.\(^{3a}\) It is probable that S-carboxamidemethylation has not any chemical influence on RGD sequence and \(\gamma\)-glutamyl group of FN. The lower binding ability of S-Cam-FN to these matrices was certainly derived from the first conformational change by S-carboxamidemethylation.

The present study indicates that S-Cam-FN may be useful as an experimental reagent to bind heparin, in addition to the suggestion that FN may fulfill physiologically yet unknown functions because it contains buried and/or inducible sites within the molecule.

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References


Heparin-Binding of Alkylated Fibronectin


